Metastasis-associated protein 1 (MTA1) is an essential downstream effector of the c-MYC oncoprotein

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The c-*myc* oncogene is among the most commonly overexpressed genes in human cancer. c-*myc* encodes a basic helix–loop–helix/leucine zipper (bHLH/LZ) transcription factor (c-MYC) that activates a cascade of downstream targets that ultimately mediate cellular transformation. Although a large number of genes are regulated by c-MYC, only a few have been functionally linked to c-MYC-mediated transformation. By expression profiling, the metastasis-associated protein 1 (MTA1) gene was identified here as a target of the c-MYC oncoprotein in primary human cells, a result confirmed in human cancer cells. MTA1 itself has been previously implicated in cellular transformation, in part through its ability to regulate the epithelial-to-mesenchymal transition and metastasis. MTA1 is a component of the Mi-2/nucleosome remodeling and deacetylating (NURD) complex that contains both histone deacetylase and nucleosome remodeling activity. The data reported here demonstrate that endogenous c-MYC binds to the genomic MTA1 locus and recruits transcriptional coactivators. Most importantly, short hairpin RNA (shRNA)-mediated knockdown of MTA1 blocks the ability of c-MYC to transform mammalian cells. These data implicate MTA1 and the Mi-2/NURD complex as one of the first downstream targets of c-MYC function that are essential for the transformation potential of c-MYC.

nucleosome remodeling and deacetylating | Mi-2 | epithelial-to-mesenchymal transition

The oncogene c-*myc* is among the most broadly overexpressed oncogenes in human cancer (1). c-*myc* encodes a transcription factor, c-MYC, that regulates the expression of downstream target genes whose products mediate the biological activities of c-*myc* (2, 3). The identity of these downstream targets remains only partially elucidated, with only a limited number of downstream genes playing well documented roles in c-MYC-mediated transformation (4–6). These targets include the genes encoding the enzymes lactate dehydrogenase-A (LDH-A) and ornithine decarboxylase (ODC). For both LDH and ODC, genetic evidence now suggests a strict requirement in the MYC transformation pathway (4–6). For two other genes, HMG-I/Y and HSP90A, indirect evidence has also suggested a role in c-MYC-mediated transformation (7, 8). Clearly, a more complete knowledge of the downstream effectors critical for c-MYC-mediated transformation will be required for a thorough understanding of the role of c-MYC in human cancer. We report here that the gene encoding metastasis-associated protein 1 (MTA1) is an essential effector of the transforming activity of c-MYC. MTA1 has previously been demonstrated to be a critical regulator of the metastatic process in both human and rodent mammary tumors (9, 10), and more recently in other tumor types as well (11–14). Current models suggest that MTA1 regulates metastatic potential as part of the multiprotein Mi-2/nucleosome remodeling and deacetylating (NURD) complex by controlling the epithelial-to-mesenchymal transition (EMT) (15, 16).

As discussed above, knowledge of the downstream targets of c-MYC that mediate its potent biological activities remains limited. Although a handful of c-MYC targets have been shown to be important for their ability to regulate cell cycle progression (17, 18) or apoptosis, only the LDH-A and ODC genes have been shown to be essential for c-MYC-mediated transformation. We therefore conducted an expression profile screen to identify novel genes essential for c-MYC-mediated transformation. The identification and verification of the Mi-2/NURD subunit MTA1 as a c-MYC target essential for transformation provides a substantial advance in our understanding of the biochemical pathways regulated by this ubiquitous human oncoprotein.

Methods

Cell Lines and Retroviral Infection. Normal human diploid fibroblasts (NHDF) used in these studies included the IMR-90 and 2091 strains (American Type Culture Collection). The immortalized rat fibroblast line Rat1a was obtained from C. Dang (The Johns Hopkins University, Baltimore) and the human breast cancer line MCF7 was obtained from American Type Culture Collection. Cell lines were maintained in 10% FBS-DMEM. The media were supplemented with 100 μg of penicillin per milliliter and 100 μg of streptomycin sulfate per milliliter. For NHDF lines, quiescence was achieved by maintaining the cells in 0.1% FBS-DMEM for 48 h, and serum stimulation was accomplished by replacing media with 10% FBS-DMEM for the indicated time. c-MYC/ER, an estrogen receptor fusion protein, was activated by adding hydroxytamoxifen (4-OHT) to growth media at a final concentration of 200 nM (Sigma). Where specified, cells were exposed to cycloheximide (CHX) at 20 μg/ml for 30 min before addition of 4-OHT. mRNA was harvested at 4 h after 4-OHT treatment.

Retroviral-mediated expression of the c-MYC/ER protein was accomplished by transfection of an MIGR1-based plasmid into the Phoenix-packaging cell line by using the calcium phosphate method. Viral supernatant was harvested 48–60 h posttransfection and used to infect NHDF cells in the presence of 8 μg/ml polybrene. After 2–3 days, infected cells were enriched by FACS for GFP. GFP-positive cells from each infection were pooled and expanded for a limited number of population doublings before use in individual experiments.

mRNA Analysis, Small Interfering RNA (siRNA)-Mediated Knockdown, and Western Blotting. mRNA was harvested from cells at the time points indicated by using the RNeasy method (Qiagen, Valencia,

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Abbreviations: LDH-A, lactate dehydrogenase-A; ODC, ornithine decarboxylase; MTA1, metastasis-associated protein 1; NURD, nucleosome remodeling and deacetylating; EMT, epithelial-to-mesenchymal transition; NHDF, normal human diploid fibroblast; 4-OHT, hydroxytamoxifen; siRNA, small interfering RNA; shRNA, short hairpin RNA; qRT-PCR, quantitative real-time RT-PCR; MbII, MYC homology box II; ER, estrogen receptor.

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CA). This mRNA was converted to cDNA by using SuperScript (Invitrogen) and subjected to quantitative real-time PCR by using the 7000 sequence detection system (Applied Biosystems Prism) and SYBR Green PCR Master Mix kit (Applied Biosystems). Primer sequences are available upon request. In all cases, mRNA levels between samples were normalized to actin levels. Enzymatic assays were performed in triplicate, and data are expressed as mean ± standard error.

siRNAs for human c-MYC and GFP were purchased from Dharmacon Research (Layfayette, CO). Cells were transfected with 200 nM of the siRNA oligos per well in six-well plates by using Oligofectamine (Invitrogen). Cells were harvested, and mRNA was extracted at 48 h posttransfection. For short hairpin RNA (shRNA)-mediated knockdown of MTA1 in Rat1a cells, oligonucleotides targeting rat MTA1 was cloned into the pSuper-retro vector (OligoEngine, Seattle, WA). The plasmids were used to generate viral supernatant as described above.

Western blots were performed as described (19). Antibodies used to detect c-MYC (Santa Cruz Biotechnology), MTA1 (Santa Cruz Biotechnology), and tubulin (Sigma) were obtained from commercial sources.

Soft Agar Growth Assay. Soft agar assays were performed by seeding 2,500 Rat1a c-MYC/ER-expressing cells in a layer of 0.3% agarose/DMEM over a layer of 0.6% agarose/DMEM in six-well plates. A top layer of liquid DMEM with or without 4-OHT (final concentration 200 nM) was added and changed every 3 days. Colony formation was assayed at day 12 by light microscopy.

Soft agar assays using primary rat embryonic kidney cells expressing E1A and activated ras have been described (20).

Plasmids and Transfection. The MIGR1-based retroviral expression cassettes were generated by insertion of the human c-*myc*/estrogen receptor ligand binding domain fusion cassette. The c-MYCΔ/ER mutant was generated by deletion of amino acids 110–150 by using the QuikChange mutagenesis protocol (Stratagene).

Tumor Induction. Mouse B cell lymphoma was generated as described (21). Briefly, bone marrow cells from p53-null mice were isolated and infected with the c-MYC/ER-encoding retrovirus described above. Infected cells were s.c. injected into C57BL/6 mice. c-MYC/ER was activated by daily i.p. injections of 1 mg of 4-hydroxytamoxifen (Sigma) resuspended in pharmacy-grade olive oil. After 3–4 weeks, all of these mice developed tumors. Tumor cells from one of these mice were harvested and transferred s.c. into four naive mice. After 9 days of 4-OHT treatment, two mice were continued on this treatment and two mice had 4-OHT withdrawn for 4 days. Tumor cells were then harvested from the mice, and MTA1 mRNA expression levels were determined by quantitative real-time RT-PCR (qRT-PCR).

Chromatin Immunoprecipitation (ChIP). For ChIP assays, NHDF cells were plated on 15-cm dishes, incubated for 24 h, and then deprived of growth factors for a subsequent 24 h by incubation in 0.1% serum-containing medium. After 0 or 2 h of serum stimulation, cells were fixed in 1% formaldehyde. Chromatin was sheared to an average size of 500–1,000 bp by sonication (6–8 times with 10-s pulses, 30% output on a Branson Model 250). Lysates corresponding to 5–10 $\times$ 10^6 cells were rotated at 4°C overnight with 2 μg of polyclonal antibodies specific for c-MYC (sc-764, Santa Cruz Biotechnology), anti-acetyl histone H3, anti-acetyl histone H4 (Upstate, Charlottesville, VA), or normal rabbit IgG. Precipitated DNA fragments were quantified by using qPCR as described above. For ChIP assays using the H1299 cell line expressing the c-MYC/ER protein, cells were treated with 4-OHT or ethanol (EtOH) for 2 h before harvesting.

Results

A screen for c-MYC targets essential for transformation was conducted by expressing the conditional c-MYC/ER (22) fusion protein in normal diploid human fibroblasts. The fusion of c-MYC to a modified version of the ligand-binding domain of the estrogen receptor allows the selective activation of c-MYC by the addition of the synthetic estrogen analog 4-OHT (23). By using this system, expression profiling was performed to identify genes whose transcription was induced by c-MYC. This screen was restricted to the identification of targets whose transcription was strictly dependent on a domain of c-MYC termed MYC homology box II (MbII). MbII resides within the transactivation domain of c-MYC and is essential for transformation of mammalian cells (24–26). The major function ascribed to MbII is the recruitment of a family of histone acetyltransferase complexes that serve as cofactors in transcription (19, 27). The identification of c-MYC targets whose transcription depends on MbII was predicted to enrich for genes whose activation is important in c-MYC-mediated transformation. To document the inability of the c-MYC ΔMbII deletion mutant to transform cells, a soft agar assay using the Rat1a cell line was used. These cells are transformed by c-MYC in the absence of cooperating oncogenes (25). As expected, activation of c-MYC/ER in Rat1a cells by 4-OHT treatment resulted in the formation of large, multicell soft agar colonies (Fig. 6, which is published as supporting information on the PNAS web site), whereas deletion of MbII completely inhibited growth in soft agar.

For the identification and validation of MbII dependent targets, NHDF were generated that express either the wild-type or ΔMbII c-MYC/ER fusion proteins described above. Cells expressing only the vector cassette served as a negative control. Similar expression of wild-type and mutant c-MYC/ER was documented by Western blotting (Fig. 1*A*). After activation of c-MYC in early passage NHDFs, mRNA was harvested and hybridized to a cDNA microarray contained 9,600 human genes. This analysis was performed in triplicate by using mRNA from independently derived pools of c-MYC/ER-expressing NHDF cells. As mentioned above, MTA1 was among the genes that exhibited a consistent pattern of MbII-dependent induction by c-MYC. As shown in Fig. 1*B*, MTA1 typically showed a 2.5-fold induction by wild-type c-MYC. This induction was blocked by deletion of the MbII domain. The level of induction observed for MTA1 is similar to that observed for cyclin D2, one of the few known MbII-dependent targets of c-MYC (28). Although this pattern of MTA1 expression was evident on multiple microarrays, verification by qRT-PCR was also performed. In this analysis, MTA1 was activated by c-MYC in an MbII-dependent manner (Fig. 1*C*). To further characterize the ability of c-MYC to activate transcription of the MTA1 gene, a kinetic analysis was performed in c-MYC/ER-expressing NHDF cells. MTA1 transcript levels increased within 4 h of c-MYC activation and continued to rise for at least 48 h (Fig. 1*D*). For comparison, induction of the known c-MYC target genes cyclin D2 and CAD was also determined (29, 30). For all three genes, kinetics and levels of induction were similar, suggesting that the responsiveness of MTA1 to c-MYC is similar to that of more well characterized c-MYC target genes.

To determine whether the induction of MTA1 by c-MYC is conserved across distinct tissues and species, we examined the ability of the c-MYC/ER protein to induce MTA1 transcription in a murine B cell lymphoma line. As shown in Fig. 1*E*, activation of c-MYC in these cells resulted in elevated MTA1 transcripts within 2 h, and levels of MTA1 continued to rise over the course of the induction regime. Although initially identified as a c-MYC target in human fibroblasts, data from the murine lymphoma system support a model in which c-MYC regulates MTA1 transcription in a variety of mammalian cell lineages.

The kinetic analysis of MTA1 induction by c-MYC in Fig. 1 *D* and *E* demonstrated that induction occurs rapidly, raising the possibility

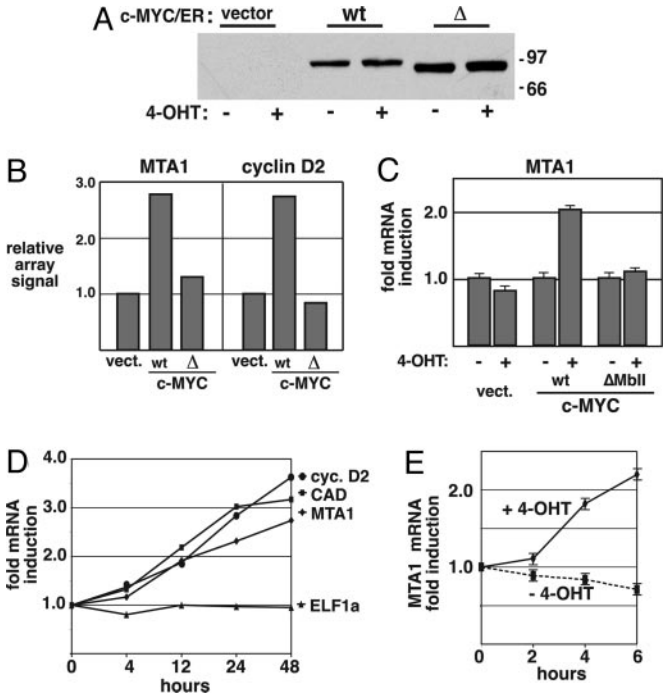

Fig. 1. Identification of the breast cancer metastasis regulator MTA1 as a transcriptional target of the c-MYC oncoprotein. (*A*) Western blot analysis shows that the wild-type and ΔMbII mutant of the human c-MYC-estrogen receptor fusion protein were comparably expressed in stable pools of NHDF cells. (*B*) Microarray analysis was performed by using mRNA harvested from cells at 4 h after 4-OHT treatment. Array signals for MTA1 and cyclin D2 are shown for the 4-OHT treated groups. (*C*) qRT-PCR was performed to verify the induction of MTA1 transcription by c-MYC. (*D*) The kinetics of MTA1 transcript induction in NHDF cells after c-MYC/ER activation was determined. As controls, levels were also determined for the c-MYC target genes cyclin D2 and CAD. As a negative control, levels of ELF1a transcript levels were quantitated. Actin levels were used to normalize expression patterns between samples. (*E*) Regulation of MTA1 by c-MYC/ER in a murine B cell lymphoma was assessed. Cells from the tumor were explanted and adapted for growth in culture. These lymphoma cells were 4-OHT-treated for the times indicated, and MTA1 transcript levels were quantitated by qRT-PCR. Nontreated cells served as a negative control, and samples were normalized for actin mRNA expression.

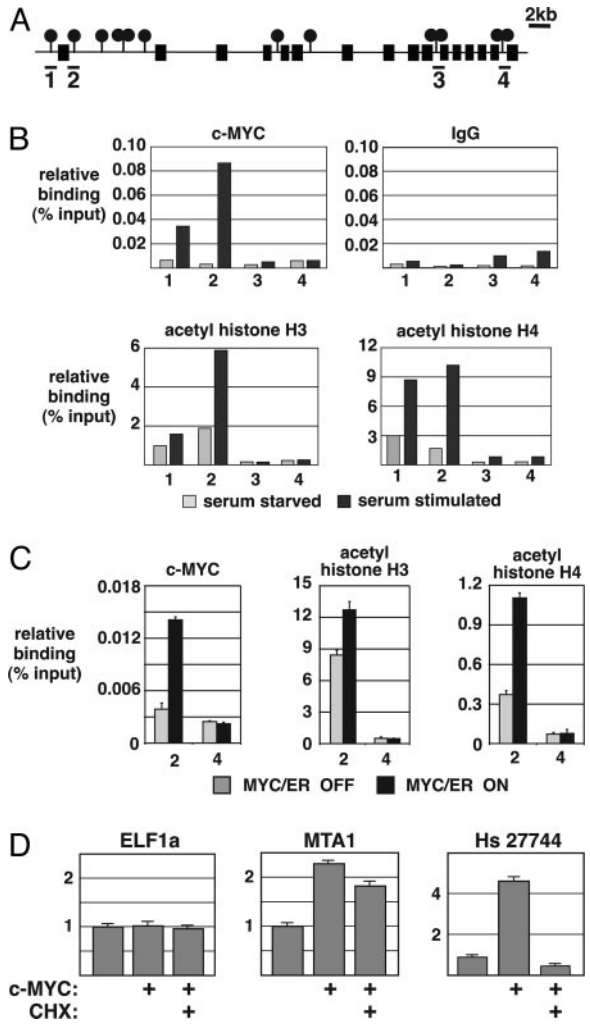

Fig. 2. *In vivo* binding of c-MYC to the endogenous MTA1 locus in human cells. (*A*) The MTA1 genomic locus contains 16 exons dispersed over 60 kb (indicated by black boxes). Within the locus, several potential matches to the c-MYC consensus binding site were identified (filled circles). (*B*) Four of these sites (sites 1–4) were selected for direct examination of c-MYC binding by using the ChIP technique. For this analysis, c-MYC was induced in growth factor-deprived NHDFs by serum stimulation for 2 h. ChIP analysis was performed to detect DNA associated with the c-MYC protein or with acetylated histones H3 or H4. Nonimmune rabbit IgG was used in control ChIP experiments. (*C*) NHDF cells expressing the c-MYC/ER protein were treated with 4-OHT for 2 h and then subjected to ChIP analysis as above. (*D*) To test whether MTA1 is a direct or indirect target of c-MYC, c-MYC/ER-expressing NHDFs were used. After growth factor starvation, cells were pretreated with CHX for 30 min, and then 4-OHT was added in the presence of CHX for 4 h to activate c-MYC/ER. Cells were harvested and levels of MTA1 transcript were determined by qRT-PCR. As controls, levels of ELF1a and Hs27744 transcripts were also determined.

that MTA1 is a primary downstream target of c-MYC rather than an indirect target whose induction requires synthesis of an intermediary. Examination of the human MTA1 genomic locus revealed the presence of 12 sites with significant homology to the consensus c-MYC binding site (CACGTG) (Fig. 2*A*). These sites are distributed across the MTA1 locus, with one present immediately upstream of the transcriptional start site, five present in intron 1, and the remainder located in introns 4, 6, 10, and 15. c-MYC-binding sites within the first intron have been shown to be important in many of its downstream targets including ODC, α-prothymosin, and nucleolin (31–33). To determine whether MTA1 is directly regulated by c-MYC, ChIP was used to assess binding of the endogenous c-MYC protein to the genomic MTA1 locus *in vivo*. For this analysis, c-MYC expression was induced by serum stimulation of growth factor-deprived NHDFs. Non serum-treated cells served as a negative control. c-MYC binding to the two most 5′ CACGTG motifs was strongly induced by serum stimulation (Fig. 2*B*). No significant increase in c-MYC binding to the dual CACGTG motifs present in regions 3 and 4 was observed. This observation is consistent with previous reports showing that c-MYC can distinguish potential binding sites at the 5′ and 3′ ends of its target gene loci (34). Control immunoprecipitates using normal rabbit IgG in place of anti c-MYC antibodies showed no significant changes at any of the four potential c-MYC-binding regions (Fig. 2*B*).

The most well characterized function of the MbII domain of c-MYC is the recruitment of two distinct families of histone acetyltransferase complexes (27, 35). The strict dependence of MTA1 transcription on the MbII domain of c-MYC suggested the possibility that MTA1 transcription requires one or more of these acetyltransferase complexes. The two major families of acetyltransferase complexes recruited by c-MYC are the human SAGA (Spt-Ada-Gcn5 acetyltransferase) complex, which preferentially acetylates histone H3 (36), and the TIP60 complex, which acetylates histone H4 (37, 38). By using antibodies that selectively recognize acetylated forms of histone H3 or H4 in the ChIP assay, we examined whether binding of c-MYC to the MTA1 locus correlated

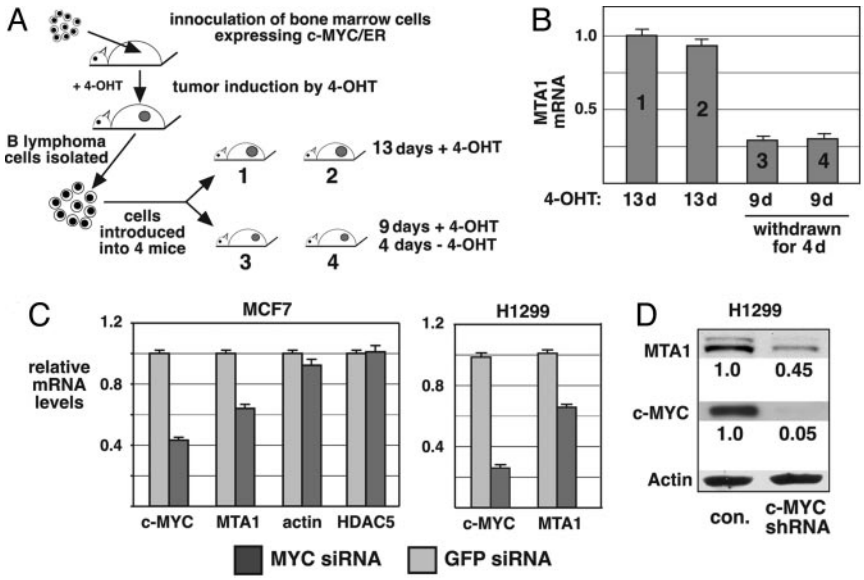

Fig. 3. MTA1 levels are regulated by c-MYC in a murine tumor model and in human breast cancer cells. (*A*) A transplantable B cell lymphoma driven by c-MYC/ER was transferred to four naive mice. (*B*) After 9 days of 4-OHT treatment, mice developed aggressively growing lymphomas. At this point, two mice were continued on 4-OHT treatment (mice 1 and 2) whereas the remaining two mice were withdrawn from treatment for 4 days (mice 3 and 4). MTA1 mRNA levels in the tumors were quantitated. (*C*) Loss of endogenous c-MYC specifically inhibits MTA1 transcription in human cancer cells. Human breast (MCF7) and lung (H1299) cancer cells were transfected with c-MYC siRNA oligos (Dharmacon) or GFP siRNA as a control. Two days after transfection, c-MYC, MTA1, actin, and HDAC5 mRNA levels were determined by qRT-PCR. Transcript levels of these genes in GFP and c-MYC siRNA-treated cells are shown, by light and dark bars, respectively. (*D*) H1299 cells were lysed 5 days posttransfection with constructs encoding either a control (con.) or c-MYC shRNA vector. Protein lysates were analyzed by Western blotting for MTA1, c-MYC, or actin, as indicated. Western blot signals for MTA1 and c-MYC were quantitated and normalized to actin levels, and numbers indicate these normalized levels.

with changes in H3 or H4 acetylation. At sites 1 and 2, dramatic increases in both histone H3 and H4 acetylation accompanied c-MYC binding (Fig. 2*B*). In contrast, acetylation at sites 3 and 4 was not significantly increased by serum stimulation. These data suggest that c-MYC binding to the MTA1 promoter is tightly correlated with the recruitment of both H3-specific and H4-specific acetyltransferase complexes. This recruitment provides an explanation for the strict requirement for the c-MYC MbII domain in MTA1 transcription. Interestingly, the levels of histone H3 and H4 acetylation at c-MYC-binding sites 1 and 2 were relatively high even before c-MYC binding, although they were still significantly increased upon c-MYC binding. A similar pre-elevation in histone acetylation levels around functional c-MYC-binding sites was recently noted in a broad survey of c-MYC bound loci (34). Overall, these data demonstrate that endogenous c-MYC selectively binds to a subset of CACGTG motifs within the endogenous MTA1 locus in native chromatin. Furthermore, c-MYC binding is linked to increased levels of histone H3 and H4 acetylation. The correlation between increased levels of acetylated histones and transcriptional activation has been established for a number of eukaryotic genes (39, 40), although the precise mechanism by which acetylation leads to transcription remains unclear.

Serum stimulation of fibroblasts leads to the induction of many transcriptional regulators beyond c-MYC. To more carefully document that the changes in chromatin observed upon serum stimulation were directly related to c-MYC, the ChIP analysis was repeated by using the c-MYC/ER system where the c-MYC protein can be selectively activated without inducing other proteins. Activation of c-MYC/ER for 2 h results in a specific increase in c-MYC binding to site 2 in the MTA1 gene (Fig. 2*C*). Concomitant with c-MYC binding, increases in the acetylation of both histones H3 and H4 were also observed. These data are analogous to those obtained for endogenous c-MYC (Fig. 2*B*).

To confirm that MTA1 is a direct downstream target of c-MYC, we again used the c-MYC/ER system, where the posttranslational activation of c-MYC by 4-OHT can be performed in the presence of protein synthesis inhibitors. This technique allows the transcription of primary c-MYC target genes. However, these transcripts cannot be translated into protein and therefore fail to propagate the cascade by activating the transcription of secondary/indirect downstream target genes. This method has been used extensively to distinguish direct and indirect targets of c-MYC (31). Treatment of cells with the protein synthesis inhibitor CHX for 30 min before c-MYC activation by 4 h of 4-OHT treatment failed to block MTA1 activation by c-MYC in NHDF (Fig. 2*D*). In contrast, activation of an indirect target of c-MYC, Hs27744, is completely blocked by CHX pretreatment. Neither 4-OHT nor CHX treatment had a significant effect on levels of ELF1a, which is not a target of c-MYC. Coupled with the direct binding of c-MYC to the MTA1 locus, these data confirm that MTA1 is directly activated by c-MYC, without a requirement for the synthesis of intermediates.

To assess whether MTA1 is induced by c-MYC during tumor formation, a mouse model was used. In this model, tumors were initiated by the infection of bone marrow cells derived from p53-null mice with a retrovirus expressing c-MYC/ER (Fig. 3*A*) (41). Bone marrow cells were inoculated s.c. into a mouse, and after several weeks of 4-OHT treatment, a B cell lymphoma developed. Cells from this lymphoma were isolated and inoculated into four naive mice. These mice were treated with 4-OHT for 9 days, during which time the injected lymphoma cells formed a rapidly growing tumor. Lymphoma mRNA was then harvested from two of the mice, whereas the remaining two mice were withdrawn from 4-OHT treatment, to inactivate c-MYC. After 4 days of c-MYC inactivation, mRNA was isolated from the tumors of the final two mice. This short inactivation of c-MYC resulted in decreased tumor growth (41). MTA1 transcript levels in the lymphomas were relatively high in the two mice with continuous c-MYC activation (Fig. 3*B*). Inactivation of c-MYC resulted in a rapid decrease in MTA1 transcript levels. These data establish that MTA1 mRNA levels are tightly linked to c-MYC activity during tumor formation *in vivo*.

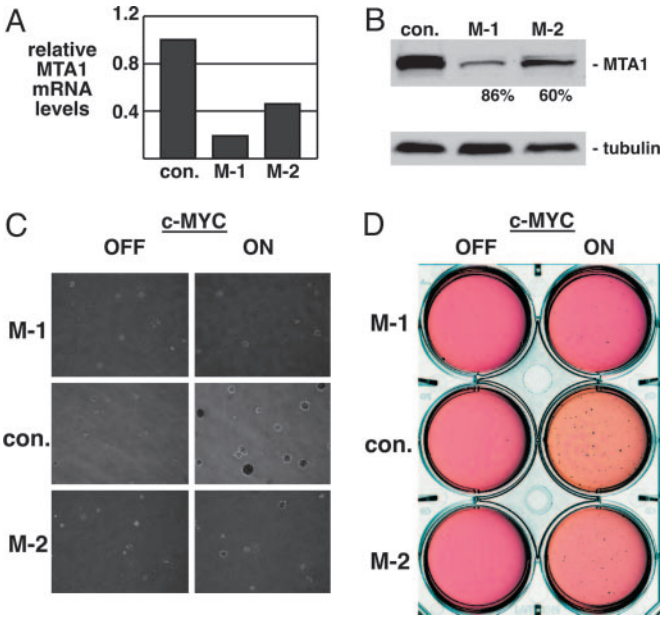

Fig. 4. MTA1 expression is essential for c-MYC-induced growth in soft agar. (*A*) Rat1a fibroblasts expressing the c-MYC/ER protein were infected with a retrovirus encoding either of two distinct shRNAs targeting MTA1. In parallel, cells were infected with a retrovirus encoding an irrelevant shRNA as a control (con.). Seventy-two hours after infection with shRNA retroviruses, mRNA was harvested from Rat1a c-MYC/ER cells and analyzed for MTA1 transcript levels. (*B*) MTA1 protein levels were determined by Western blotting of cell lysates after knockdown in Rat1a c-MYC/ER cells. Blots were probed for tubulin as a control for protein loading. (*C*) After MTA1 knockdown, Rat1a c-MYC/ER cells were treated with 4-OHT to activate c-MYC and then analyzed for growth in soft agar. Mock-treated cells served as a control. Images show colony size at 7 days after c-MYC activation. (*D*) Low magnification images of soft agar assay wells.

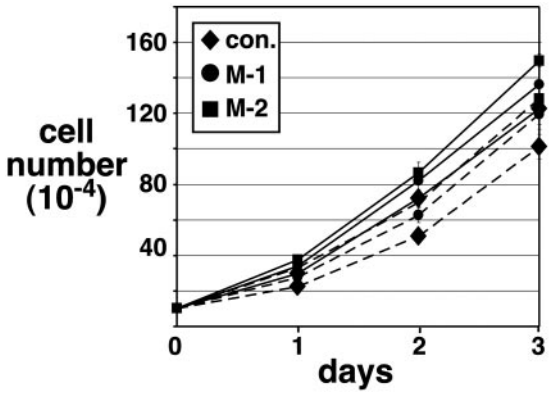

Fig. 5. MTA1 expression is not essential for c-MYC-induced cell cycle progression. Rat1a cells expressing c-MYC/ER were infected with retroviral stocks encoding the two distinct MTA1 shRNA constructs or a control shRNA as indicated. After selection for infected cells, cells were plated in the presence (solid lines) or absence (dashed lines) of 4-OHT. Cell numbers were determined by direct counting of triplicate wells at each of the time points indicated.

The importance of MTA1 expression in regulating metastasis has been most well established in breast cancer cells (42), where MTA1 was originally identified. We therefore examined whether endogenous c-MYC regulates expression of the endogenous MTA1 gene in the human breast cancer cell line MCF7. Treatment of MCF7 cells with siRNA directed against c-MYC resulted in a 60% decrease in c-MYC transcript levels (Fig. 3*C*). Examination of MTA1 levels in MCF7 cells after c-MYC knockdown demonstrated that loss of c-MYC resulted in a 40% decrease in MTA1 transcript levels. Transcript levels for several control genes examined in parallel, including actin and HDAC5, were not affected by c-MYC knockdown (Fig. 3*C*). In addition to breast, MTA1 has been demonstrated to be important in the regulation of metastatic potential in other epithelial and nonepithelial tissues (11, 13, 14, 43, 44). We therefore examined the human lung epithelial tumor line H1299 and NHDF for c-MYC-dependent transcription of MTA1. As observed in MCF7 cells, knockdown of c-MYC in H1299 and NHDF cells resulted in a significant decrease in MTA1 transcript levels (Fig. 3*C* and data not shown). Concomitant with decreased MTA1 mRNA levels after c-MYC knockdown, MTA1 protein levels were also decreased significantly (Fig. 3*D*). Taken together, these results demonstrate that the endogenous c-MYC oncoprotein directly controls transcription of the metastasis regulator MTA1 in a variety of human cell types.

Because MTA1 has been implicated as critical for several steps in the transformation process, studies were designed to assess whether its induction by c-MYC is essential for c-MYC-mediated transformation. For this analysis, the c-MYC/ER-expressing Rat1a fibroblast line discussed above was infected with retroviral stocks encoding either of two shRNAs directing knockdown of endogenous MTA1. This treatment led to a 60–86% decrease in MTA1 mRNA (Fig. 4*A*) and protein (Fig. 4*B*). In control cells, activation of c-MYC/ER led to the formation of large colonies in soft agar within 7 days (Fig. 4 *C* and *D*). In cells where MTA1 levels were knocked down, activation of c-MYC failed to induce soft agar colony formation. The requirement for MTA1 expression in transformation does not seem to be universal because primary rat embryonic kidney cells transformed with E1A and an activated allele of the ras oncogene (20) are not inhibited by MTA1 knockdown (Fig. 7, which is published as supporting information on the PNAS web site).

The failure of c-MYC to induce soft agar colony formation when MTA1 was knocked down might result from a general requirement for MTA1 in cell cycle progression, rather than a specific requirement in the transformation process. To test for a potential role of MTA1 in cell cycle progression, proliferation rates for the Rat1a c-MYC/ER cells were determined. In Rat1a cells where c-MYC/ER was not activated, knockdown of MTA1 had no inhibitory effect on cell proliferation (Fig. 5). Similarly, whereas activation of c-MYC/ER had the expected result of increasing the rate of proliferation, loss of MTA1 failed to inhibit proliferation. Therefore, the essential role played by MTA1 in c-MYC-mediated transformation is not due to an effect on cell cycle progression, but instead is due to a function specifically related to the transformation process. The fact that two separate shRNAs targeting distinct regions of the MTA1 transcript both block c-MYC-mediated transformation strongly suggests that this is a specific effect on MTA1 rather than on another, unknown target. Collectively, these results implicate MTA1 induction by c-MYC as an essential event in the process by which cells acquire the ability to form colonies in a nonadherent setting.

Discussion

The MTA1 gene was isolated based on its induction in metastatic mammary adenocarcinoma cells (10). In breast/mammary cancer, MTA1 regulates the EMT pathway at multiple points (42). Even modest alterations in MTA1 levels have a profound effect on the invasive growth of transformed cells (45). Remarkably, little is known of what lies upstream of MTA1 to regulate its expression. This study provides a mechanistic insight into the control of the metastasis regulator MTA1 by demonstrating that it is a direct transcriptional target of the c-MYC oncoprotein. The evidence linking c-MYC to MTA1 came initially from an unbiased genetic screen and was subsequently verified by empirical approaches. These approaches included demonstrating

that endogenous c-MYC binds and regulates the endogenous MTA1 gene. In support of the model in which c-MYC regulates the EMT by activating transcription of MTA1, c-MYC activation ultimately leads to increased levels of the SNAIL repressor (data not shown), a protein that lies downstream of MTA1 in the EMT pathway (42). In mice, tumors induced by expression of a conditional allele of c-MYC expressed high levels of MTA1. These levels dropped sharply when c-MYC activity was turned off. Most importantly, blocking the ability of c-MYC to induce MTA1 transcription completely blocked transformation of cells in a classical soft agar assay, without affecting their rate of proliferation when grown adherently. This result suggests that MTA1 induction by c-MYC provides a function necessary only when cells grow in nonadherent conditions. Growth in nonadherent conditions has been repeatedly shown to provide an accurate reflection of cellular transformation and tightly correlates with the ability of cells to form aggressive tumors in animals. To our knowledge, the only known targets of c-MYC that have documented roles in c-MYC-mediated transformation are the LDH-A and ODC genes (4–6). In fact, the induction of LDH-A by c-MYC may explain the "Warburg effect," which is the capacity of tumor cells to grow in anaerobic conditions while their normal parental cells cannot (46). Although other targets such as cyclins D1 and D2 and cyclin-dependent kinase 4 (cdk4) are important for the ability of c-MYC to increase the rate of cell cycle progression (17, 18), only LDH-A (4, 5) and now MTA1 have been shown to play a specific role in the transformation process without generically affecting cell cycle progression.

It is of interest to note that Mazumdar *et al*. (47) have reported that MTA1 expression in MCF7 cells is sufficient to induce invasive growth. Overexpression of MTA1 in the Rat1a fibroblast line in contrast failed to increase invasive growth (data not shown). These data suggest that there may be a tissue-specific component to the role played by MTA1 in cellular transformation.

Although the link between MTA1 and the EMT remains controversial, it is tempting to speculate that c-MYC might participate in the metastatic process through its direct regulation of MTA1. Further studies on the cellular and biochemical functions of MTA1 will be required to address this issue. It will also be of interest to define the precise biochemical activity that is provided by induction of MTA1 levels, an activity that will presumably be related to the role of MTA1 in the Mi-2/NURD deacetylase complex. Ultimately, the identification of the Mi-2/NURD targets affected by c-MYC's activation of MTA-1 may provide useful therapeutic strategies.

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